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# Low-Concentration Kinetics of Atmospheric $CH_4$ Oxidation in Soil and Mechanism of $NH_4^+$ Inhibition

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**$NH_4^+$ inhibition kinetics for $CH_4$ oxidation were examined at near-atmospheric $CH_4$ concentrations in three upland forest soils. Whether $NH_4^+$-independent salt effects could be neutralized by adding nonammoniacal salts to control samples in lieu of deionized water was also investigated. Because the levels of exchangeable endogenous $NH_4^+$ were very low in the three soils, desorption of endogenous $NH_4^+$ was not a significant factor in this study. The $K_{m(app)}$ values for water-treated controls were 9.8, 22, and 57 nM for temperate pine, temperate hardwood, and birch taiga soils, respectively. At $CH_4$ concentrations of ≤15 μl liter$^{-1}$, oxidation followed first-order kinetics in the fine-textured taiga soil, whereas the coarse-textured temperate soils exhibited Michaelis-Menten kinetics. Compared to water controls, the $K_{m(app)}$ values in the temperate soils increased in the presence of $NH_4^+$ salts, whereas the $V_{max(app)}$ values decreased substantially, indicating that there was a mixture of competitive and noncompetitive inhibition mechanisms for whole $NH_4^+$ salts. Compared to the corresponding $K^+$ salt controls, the $K_{m(app)}$ values for $NH_4^+$ salts increased substantially, whereas the $V_{max(app)}$ values remained virtually unchanged, indicating that $NH_4^+$ acted by competitive inhibition. Nonammoniacal salts caused inhibition to increase with increasing $CH_4$ concentrations in all three soils. In the birch taiga soil, this trend occurred with both $NH_4^+$ and $K^+$ salts, and the slope of the increase was not affected by the addition of $NH_4^+$. Hence, the increase in inhibition resulted from an $NH_4^+$-independent mechanism. These results show that $NH_4^+$ inhibition of atmospheric $CH_4$ oxidation resulted from enzymatic substrate competition and that additional inhibition that was not competitive resulted from a general salt effect that was independent of $NH_4^+$.**

Atmospheric $CH_4$ contributes substantially to the greenhouse effect, and the concentration of atmospheric $CH_4$ has increased dramatically in the past century because of human activity associated with agriculture, land use changes, and industry (34, 35). Bacterial oxidation of atmospheric $CH_4$ in well-drained soils is an important regulator of atmospheric $CH_4$ concentration, yet the organisms responsible remain unidentified and the physiology of the process is poorly understood (9, 35, 36). Although soil $CH_4$ consumption is inhibited by a wide variety of anthropogenic disturbances, such as agriculture, N deposition, and forestry (12, 17, 22, 23, 32, 43, 44), predictable inhibition patterns have failed to emerge, which has made it difficult to predict the effects of disturbance on soil $CH_4$ flux in various ecosystems. The most commonly reported disturbance effect is that of $NH_4^+$ fertilizers, which can suppress soil $CH_4$ consumption by up to 70% (1, 8, 10, 17, 22, 32, 33, 37, 38, 43). In the field, inhibition may occur immediately following fertilization, may be delayed for months to years, or may never occur despite years of chronic fertilization (9, 17). This variety of responses may stem at least in part from the distribution of physiologically diverse methane oxidizer populations across sites (17, 18, 20).

Of the various $NH_4^+$ inhibition patterns, immediate inhibition is the best documented. As in field studies, however, physiological laboratory studies have produced variable results, suggesting that there may be multiple inhibition mechanisms (15, 17, 26–28, 36, 39). Physicochemical similarities between $CH_4$ and $NH_3$ may permit these two compounds to compete for enzyme active sites so that fortuitous $NH_3$ oxidation competitively inhibits $CH_4$ oxidation (38). Although this mechanism has been demonstrated to occur in pure cultures of methanotrophic bacteria (6) and in a $CH_4$-producing agricultural soil (15), it has not been demonstrated to occur in well-drained, nonagricultural mineral soils, which comprise the dominant terrestrial sink for atmospheric $CH_4$ (14, 38, 45), nor has it been demonstrated to occur at near-atmospheric $CH_4$ concentrations. In many cases, the kinetics of immediate $NH_4^+$ inhibition in soil cannot be reconciled easily with substrate competition (15, 16, 26–28, 39). An alternative mechanism has been proposed, whereby the toxicity of $NO_2^-$ or $NH_2OH$ produced by fortuitous $NH_4^+$ oxidation suppresses methanotrophic activity (26, 27, 39). Hence, multiple inhibition mechanisms may be involved, and these mechanisms may vary with the physiology of different $CH_4$ oxidizer populations (17).

Two physiologically distinct communities of $CH_4$ oxidizers apparently exist in soil. One group, generally associated with atmospheric $CH_4$ consumption, exhibits an extremely high affinity for $CH_4$. Representatives of this group have yet to be cultivated or otherwise identified (9). The second group exhibits a much lower affinity for $CH_4$ and is generally associated with common methanotrophs, such as those that have been studied in pure culture for many years (2, 9). In upland mineral soils, only high-affinity activity is usually detectable without artificial enrichment with high $CH_4$ concentrations in the laboratory. However, the only prior study in which kinetic constants for $NH_4^+$ inhibition of soil $CH_4$ oxidation were reported was conducted in a periodically moist, organic matter-rich agricultural soil with demonstrable methanogenesis (15, 16). Such a soil potentially harbors a rich community of $CH_4$ oxidizers representing a continuum from low-affinity organisms to high-affinity organisms. Although this important investigation

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TABLE 1. Sampling sites and characteristics of the soils examined in this study

| Ecosystem type (dominant plant) | Location | Soil texture | Concn of extractable $NH_4^+$ (mg of N kg of dry soil$^{-1}$)[a] | Water-holding capacity (g of $H_2O$ g of dry soil$^{-1}$) |
|---|---|---|---|---|
| Birch taiga (*Betula papyrifera*) | University of Alaska, Fairbanks | Fine silt | 0.11 (0.014)[b] | 0.62 |
| Temperate hardwood (*Quercus velutina*) | Harvard Forest, Petersham, Mass. | Sandy loam | 6.98 (2.91)[c] | 0.84 |
| Temperate pine (*Pinus resinosa*) | Harvard Forest, Petersham, Mass. | Sandy loam | 7.83 (2.49)[c] | 0.73 |

[a] concentration of $NH_4^+$ N in the upper 10 cm of the mineral horizon, which includes the zone of maximum $CH_4$ oxidation. The values in parentheses are standard deviations.
[b] Data from this study. The concentration of extractable $NH_4^+$ was determined as described previously (17).
[c] Data from A. Magill (29a). See reference 30 for more information.

demonstrated that $NH_4^+$ inhibits $CH_4$ oxidation via enzymatic substrate competition in an agricultural humisol, it is unclear to what extent its results apply to well-drained mineral soils lacking endogenous $CH_4$ sources. Physiological studies of soil $CH_4$ oxidation typically derive kinetic constants from oxidation rates at $CH_4$ concentrations ranging from atmospheric levels ($\sim$1.7 μl liter$^{-1}$) to $\gg K_m$ for high-affinity $CH_4$ oxidizers. Even in soil in which only high-affinity organisms are active, the $CH_4$-oxidizing enzyme(s) could respond differently to $NH_4^+$ at high $CH_4$ concentrations than at near-atmospheric concentrations (15, 39). Thus, to study $NH_4^+$ inhibition of high-affinity $CH_4$ oxidizers per se, it would be preferable to examine inhibition kinetics at near-atmospheric $CH_4$ concentrations in a soil with no apparent endogenous $CH_4$ source.

A common shortcoming of $NH_4^+$ inhibition studies, regardless of the organisms involved, has been a lack of attention to nonammoniacal salt effects despite numerous reports of substantial inhibition by such salts (1, 10, 15, 17, 24). King and Schnell (28) examined the effects of several $Cl^-$ and $SO_4^{2-}$ salts and concluded that nonammoniacal salts indirectly inhibit $CH_4$ oxidation by desorbing endogenous $NH_4^+$ from cation exchange sites in the soil, which then directly inhibit $CH_4$ oxidation. Many N-limited soils, however, have extremely low concentrations of exchangeable $NH_4^+$, yet are substantially inhibited by nonammoniacal salts (17), suggesting that these salts have $NH_4^+$-independent effects on atmospheric $CH_4$ oxidizers. Additional mechanisms may alter inhibition kinetics, thus hindering the diagnosis of $NH_4^+$-specific inhibition.

With the limitations described above in mind, we used a simple steady-state kinetics approach to assess the mechanism of $NH_4^+$ inhibition of $CH_4$ oxidation at near-atmospheric concentrations (1.8 to 15 μl liter$^{-1}$) in three well-drained, N-limited forest soils that lack known endogenous $CH_4$ sources. In addition, we examined the effects of nonammoniacal salts in parallel samples to judge the utility of these salts as experimental controls for neutralizing $NH_4^+$-independent salt effects.

## MATERIALS AND METHODS

**Field sites.** We studied soils from two temperate forests and one taiga forest, the major characteristics of which are listed in Table 1. The two temperate soils were from the Harvard Forest Long-Term Ecological Research site in western Massachusetts (29), where fertilizer inhibition of atmospheric $CH_4$ consumption was first observed (43). The sites and their biogeochemical cycles have been described in detail previously (7, 8, 30). The taiga site is approximately 120 years postburn, and the understory is dominated by *Rosa acicularis* and *Equisetum* spp. The mineral soil consists of a uniform layer of silty glacial loess. The pedology, ecology, and biogeochemistry of this site are similar to the pedology, ecology, and biogeochemistry of nearby sites that have been described previously (17). All three sites are well drained and have never been observed to produce $CH_4$ (7, 8, 19).

**Soil processing and bioassays.** All experiments were performed at the University of Alaska, Fairbanks. At each site, soil was collected in bulk from the upper 10 cm of the mineral soil, which included the zone of maximum $CH_4$ oxidation, and stored in perforated plastic bags for transport to the laboratory. The soil was homogenized by sieving it through a 4-mm-mesh screen. The water-holding capacity of each soil type was determined as described previously (18), and the moisture was adjusted so that the final water content was 30 to 35% of the water-holding capacity (Table 1) after the final treatment with deionized water or salt solutions. This moisture level was determined previously to be optimal for atmospheric $CH_4$ consumption in a wide variety of soils (18). Samples were treated with deionized water, $K_2SO_4$, $(NH_4)_2SO_4$, $Na_2SO_4$ (taiga soil only), KCl, or $NH_4Cl$. Each salt solution was added to a single bulk sample (0.1 ml g of dry soil$^{-1}$), which was then mixed thoroughly and subdivided into individual samples. The salts were added at a rate of 5.6 μmol of cations per g of dry soil, so that all of the salts were equinormal with respect to cations. The resulting $NH_4^+$ additions were equivalent to 75 mg of N per kg of dry soil, which matched the treatments used in previous experiments (17). This amount of $NH_4^+$ was intended to overwhelm the endogenous soil N (Table 1) yet remain within the range of soil $NH_4^+$ concentrations reported for forest soils with various land use histories (13, 31, 42).

For each treatment, 12 subsamples (10 g of dry soil) were placed in 70-ml serum vials sealed with butyl rubber septa and allowed to equilibrate overnight. The following morning the vials were equilibrated with laboratory air ($\sim$1.8 μl of $CH_4$ liter$^{-1}$) and sealed, and their headspace $CH_4$ concentrations were adjusted by injecting appropriate volumes of 1% $CH_4$ premixed with air (Scott Specialty Gases, Plumsteadville, Pa.); the headspace $CH_4$ concentrations tested were approximately 1.8 (no $CH_4$ added), 5, 10, and 15 μl liter$^{-1}$. Three replicates for each treatment at each $CH_4$ concentration were prepared. For the temperate soils, a single 2-h $CH_4$ oxidation assay was carried out with the headspace $CH_4$ concentration measured at the beginning and the end of the assay. The resulting consumption rate ($d[CH_4]/dt$) was paired with the corresponding midpoint $CH_4$ concentration in order to obtain a plot of oxidation rate versus $CH_4$ concentration (Fig. 1b and c). For the birch taiga soil, a modified procedure was used because oxidation was 2 orders of magnitude slower in this soil than in the temperate soils (Table 2). On the first day of the experiment, a 3.3-h assay was carried out with the headspace $CH_4$ concentration measured at the beginning and the end of the assay. The samples were kept sealed and were allowed to consume $CH_4$ overnight, and the 3.3-h assay was repeated on the second day and again on the third day. Identical assays were then repeated every 48 h until either a threshold concentration was established or through the ninth day, whichever occurred first. As with the temperate soils, the rate ($d[CH_4]/dt$) from each 3.3-h assay was plotted against the corresponding midpoint $CH_4$ concentration in order to obtain a plot of oxidation rate versus $CH_4$ concentration (Fig. 1a). $CH_4$ was analyzed by gas chromatography as described previously (5, 17, 18).

Because $Cl^-$ inhibited $CH_4$ oxidation much more than did $SO_4^{2-}$, the effects of $Cl^-$ and $SO_4^{2-}$ salts on general microbial respiration in the birch taiga soil were examined. The amount of $CO_2$ that accumulated was determined by measuring headspace $CO_2$ concentrations, which never exceeded 2%, at the beginning and end of a 1-week incubation period. The amount of $CO_2$ that accumulated in each salt treatment was compared to the amount of $CO_2$ in water-treated controls. $CO_2$ was analyzed by gas chromatography as described previously (5, 18).

**Statistical analyses and calculations.** The effects of the salt treatments and $CH_4$ concentration on oxidation rates in each soil were analyzed by analysis of covariance by using treatment as the independent factor and the initial $CH_4$ concentration as a covariate; Bonferroni contrasts were used in multiple comparisons. Because the incubation times were the same for all treatments in a given soil, the treatments with higher oxidation rates consumed more substrate than the treatments with lower oxidation rates. For regression analyses, therefore, the oxidation rate from an individual assay was paired with the corresponding midpoint $CH_4$ concentration (Fig. 1) rather than the initial concentration. This standard technique normalizes consumption rates for unequal substrate concentrations among treatments and also minimizes the deviation from standard Michaelis kinetics that can result from substrate depletion (41). First-order kinetics were modeled by linear regression, and the rate constants were esti-

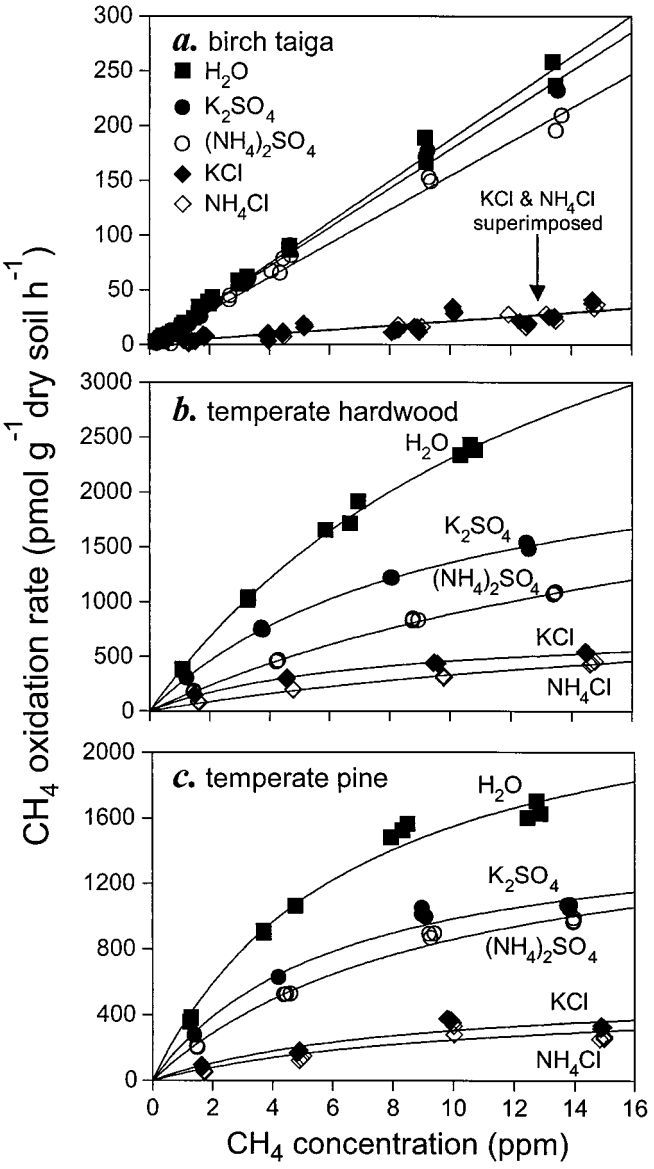


FIG. 1. $CH_4$ oxidation kinetics in three upland forest soils, a birch taiga soil (a), a temperate hardwood soil (b), and a temperate pine soil (c). The data for the birch taiga soil are shown with linear regression lines, whereas the data for the temperate soils are shown with nonlinear regression curves fit to the Michaelis-Menten equation. Each point represents a single rate measurement.

mated from the slope of the regression line, with both variables expressed in picomoles. Michaelis constants were obtained from a least-squares nonlinear regression fit of the data to the Michaelis-Menten equation. For treatments exhibiting Michaelis kinetics, pseudo-first-order rate constants were calculated as $V_{max}/K_m$, with both constants expressed in picomoles. Relative inhibition was calculated for each treatment as follows: relative inhibition = $(1 - k_2/k_1) \times 100$, where $k_1$ is the first-order (or pseudo-first-order) rate constant for the control sample and $k_2$ is the first-order (or pseudo-first-order) rate constant for the treated sample.

Examining the relationship between relative inhibition and $CH_4$ concentration requires calculating inhibition ratios for two treatments at specific $CH_4$ concentrations. In doing this, care must be taken not to compare rates derived from substantially different midpoint $CH_4$ concentrations, even when the initial concentration is the same for all treatments. This problem arises when a faster sample consumes substantially more substrate than a slower sample, resulting in a disparity between midpoint $CH_4$ concentrations in the two assays. For this reason, the method used to calculate relative inhibition at specific $CH_4$ concentrations varied according to the relative rates among treatments and the type of kinetics involved for each soil. In the birch taiga soil, which displayed first-order kinetics, inhibition ratios were calculated directly from the rates measured in the experiments on the first day of incubation. Because the oxidation rates were very low in this soil, the differences in midpoint $CH_4$ concentrations among the treatments were trivial. The inhibition by each salt compared to the deionized water control was calculated for each of the initial $CH_4$ concentrations (~1.8, 5, 10, and 15 μl liter$^{-1}$) and plotted against the midpoint $CH_4$ concentration occurring in the control (Fig. 2a). In the temperate soils, which displayed Michaelis kinetics, the rates were high, and the midpoint $CH_4$ concentrations varied among the treatments. Hence, estimated inhibition ratios were calculated by entering four different $CH_4$ concentrations (1.8, 5, 10, and 15 μl liter$^{-1}$) into the regression equation obtained for each treatment. The calculated oxidation rates at each concentration were then used to calculate inhibition ratios for each salt compared to the water control. Each ratio was then plotted against the $CH_4$ concentration from which it was derived (Fig. 2b and c). The slope of the relationship between relative inhibition and $CH_4$ concentration was estimated by linear regression.

## RESULTS

**Birch taiga soil.** In the birch taiga soil, the $CH_4$ oxidation kinetics at concentrations of ≤15 μl liter$^{-1}$ were approximately first order ($R^2 > 0.99$ except for $Cl^-$ salts) for all treatments, but the rate constants varied among treatments (Fig. 1a; Table 2). Analyses at higher $CH_4$ concentrations (10 to 800 μl liter$^{-1}$) (data not shown) yielded a $K_{m(app)}$ for oxidation in this soil of 39 μl liter$^{-1}$ (57 nM in solution), which is typical for upland soils (2, 3, 36, 46, 47). Neither $K_2SO_4$ nor $Na_2SO_4$ significantly inhibited $CH_4$ oxidation compared to deionized water ($P = 0.78$), and the curves for $K_2SO_4$ and $Na_2SO_4$ were indistinguishable ($P = 0.91$) ($Na_2SO_4$ data not shown). Specific $NH_4^+$ inhibition, calculated using $K_2SO_4$ as the control, was relatively weak (19%) but was statistically significant ($P = 0.04$). Both KCl and $NH_4Cl$ inhibited $CH_4$ oxidation severely (~90%; slopes were significantly different from zero at $P < 0.01$) (Fig. 1a). Unlike the comparison of $K_2SO_4$ and $(NH_4)_2SO_4$, the effects of KCl and $NH_4Cl$ were indistinguishable ($P = 0.84$) (Fig. 1a). All four salts caused relative inhibition to increase as $CH_4$ concentration increased (Fig. 2a). The slopes of the increases were similar for all salts regardless of which cation was added and regardless of the final soil $NH_4^+$ concentration. All salts inhibited total microbial respiration, but like $CH_4$ oxidation, $CO_2$ production was more sensitive to $Cl^-$ salts than to $SO_4^{2-}$ salts; the relative inhibition was ~18% for both $K_2SO_4$ and $(NH_4)_2SO_4$, whereas it was 22 to 25% for KCl and $NH_4Cl$.

**Temperate forest soils.** The relative inhibition patterns for the various salts in the temperate hardwood and pine forest soils were similar to the patterns in the birch taiga soil, except that $CH_4$ oxidation conformed well to Michaelis-Menten kinetics ($R^2 > 0.98$ in most cases) (Fig. 1b and c; Table 2). With minor differences in magnitude, the pine soil exhibited the same patterns as the hardwood soil. As in the birch taiga soil, the $Cl^-$ salts were the most inhibitory salts, followed by $(NH_4)_2SO_4$ and then $K_2SO_4$. $K_2SO_4$ inhibition and specific $NH_4^+$ inhibition (relative to $K^+$) were stronger in the temperate soils than in the taiga soil; the levels of specific $NH_4^+$ inhibition in the temperate hardwood and pine soils were 54 and 34%, respectively (Table 2). As in the birch taiga soil, inhibition of $CH_4$ oxidation increased with the $CH_4$ concentration when $K^+$ salts were added. Unlike the taiga soil, however, inhibition in the temperate soils decreased as $CH_4$ concentration increased when $NH_4^+$ salts were added (Fig. 2b) (pine forest results not shown). When specific $NH_4^+$ inhibition was calculated using $K^+$ salts as controls, inhibition decreased sharply from ~50% in the presence of 1.8 μl of $CH_4$ liter$^{-1}$ to ~20 to 30% in the presence of 15 μl of $CH_4$ liter$^{-1}$ in the temperate hardwood soil (Fig. 2c).

The Michaelis parameters $K_m$ and $V_{max}$ exhibited similar patterns of responses to the various treatments in the two

temperate soils (Table 2). In deionized water controls, the $K_{m(app)}$ values were 15 and 6.7 μl liter$^{-1}$ (22 and 9.8 nM in solution) in the hardwood and pine soils, respectively. In the hardwood soil, the values of both $K_m$ and $V_{max}$ were about double the corresponding values in the pine soil, so the pseudo-first-order rate constants ($V_{max}/K_m$) were similar in the two soils (Table 2). $K^+$ salts (irrespective of the anions) either decreased or had no effect on $K_{m(app)}$ values compared to water controls, whereas $NH_4^+$ salts always increased the $K_{m(app)}$. In contrast, $V_{max(app)}$ values decreased similarly in the presence of both $K^+$ and $NH_4^+$ salts. Compared to $K^+$ salts, however, $NH_4^+$ salts increased $K_{m(app)}$ but had no effect on $V_{max(app)}$.

## DISCUSSION

Often, soil $CH_4$ oxidation at near-atmospheric $CH_4$ concentrations follows first-order reaction kinetics (3, 39, 46), as was the case in the birch taiga soil in this study (Fig. 1a). In fine-textured soils, first-order kinetics at lower $CH_4$ concentrations may result from restricted gas diffusion from the atmosphere into the soil, a purely first-order process (14, 37, 45). The birch taiga soil studied is a fine silt soil and therefore strongly limits gas diffusion from the atmosphere to the $CH_4$ oxidizers (14, 37), thus possibly increasing the $K_{m(app)}$ and creating a problem for studying low-concentration $CH_4$ oxidation kinetics in this soil (Fig. 3). By contrast, the two temperate forest soils studied have a coarse sandy texture, which enhances $CH_4$ diffusion, allowing uptake kinetics to reflect enzyme activity more closely and permitting standard kinetic analyses of $NH_4^+$ and salt inhibition of $CH_4$ oxidation at near-atmospheric $CH_4$ concentrations. The maximum $CH_4$ concentration used in our experiments (15 μl liter$^{-1}$) was similar to the $K_{m(app)}$ values in the temperate forest soils. The regression curves resulting from the kinetic analyses provided very good fits to the actual data (generally, $R^2 > 0.98$) (Fig. 1; Table 2), indicating that the kinetic models which we used accurately described the process as measured in this study.

Although there have been numerous reports of steady-state kinetic constants for soil $CH_4$ oxidation (2–4, 15, 36, 46, 47), only one previous study reported kinetic parameters for $NH_4^+$ inhibition (15). The soil studied previously was an agricultural humisol with an organic matter content of ~70% and demonstrable methanogenic activity (15, 16). These conditions probably supported a much different $CH_4$ oxidizer community than the community expected in upland mineral soils that lack an endogenous $CH_4$ source. Indeed, the $K_{m(app)}$ values for our temperate forest soils (Table 2) were substantially lower than the $K_{m(app)}$ values reported in the previous study (15), suggesting that there were physiological differences in the $CH_4$ oxidizer communities in the upland mineral soils and the agricultural humisol. In the present study we focused specifically on high-affinity $CH_4$ oxidation in three non-$CH_4$-producing upland soils from two North American biomes, subarctic taiga forest and northeastern temperate forest.

**Salt effects.** Interpreting inhibition mechanisms based on kinetic parameters in a system that is as biologically and chemically complex as soil requires careful consideration of how ions added to the system may affect the process of interest, both directly and indirectly (15, 28). All of the ions used in this study potentially could affect $CH_4$ oxidation in three basic ways. First, they could change the soil osmotic potential and impose water stress on the microbial community (18, 40); second, they could affect ion exchange, thereby altering $NH_4^+$ availability (28); and third, they could affect the $CH_4$ oxidizers directly in a number of ways (11, 15, 21). Any of these factors could alter $CH_4$ oxidation rates and kinetics and thus affect the interpretation of the specific $NH_4^+$ inhibition mechanism.

With the salt additions used in this study, the water potential in the birch taiga soil was approximately −0.2 MPa, which is the optimum water potential for atmospheric $CH_4$ oxidation in a wide variety of upland soils (18, 40). Because the birch taiga soil had the lowest water-holding capacity of the soils used in this study (i.e., the lowest water/salt ratio [Table 1]), its osmotic potential should have been the most sensitive to the salt additions. Thus, salt-related inhibition of atmospheric $CH_4$ oxidation in this study did not appear to be related to water stress.

TABLE 2. $CH_4$ oxidation kinetics in three upland forest soils

| Soil | Treatment | $K_{m(app)}$ (μl liter$^{-1}$)[a] | $V_{max(app)}$ (pmol g$^{-1}$ h$^{-1}$) | First-order rate constant (h$^{-1}$)[b] | | % Inhibition compared to[c]: | | Implied inhibition mechanism | Curve fit ($R^2$)[d] |
|---|---|---|---|---|---|---|---|---|---|
| | | | | True | Pseudo | $H_2O$ | $K^+$ | | |
| Birch taiga | $H_2O$ | | | 0.00711 | | | | | 0.994 |
| | $K_2SO_4$ | | | 0.00653 | | 8.2 | | ? | 0.996 |
| | $(NH_4)_2SO_4$ | | | 0.00526 | | 26* | 19* | ? | 0.992 |
| | KCl | | | 0.00078 | | 89* | | ? | 0.772 |
| | $NH_4Cl$ | | | 0.00072 | | 90* | 7.7 | ? | 0.771 |
| Temperate hardwood | $H_2O$ | 15.2 (1.3) | 5,807 (329) | | 0.705 | | | | 0.998 |
| | $K_2SO_4$ | 9.8 (0.4) | 2,662 (584) | | 0.510 | 28* | | Uncompetitive | 0.999 |
| | $(NH_4)_2SO_4$ | 22.4 (1.8) | 2,870 (157) | | 0.235 | 67* | 54* | Mixed competitive | 0.998 |
| | KCl | 7.2 (0.6) | 786 (30) | | 0.200 | 72* | | Uncompetitive | 0.993 |
| | $NH_4Cl$ | 25.6 (4.9) | 1,174 (155) | | 0.085 | 88* | 58* | Mixed competitive | 0.991 |
| Temperate pine | $H_2O$ | 6.7 (0.9) | 2,594 (160) | | 0.715 | | | | 0.984 |
| | $K_2SO_4$ | 6.1 (0.9) | 1,591 (98) | | 0.485 | 32* | | Noncompetitive | 0.978 |
| | $(NH_4)_2SO_4$ | 9.8 (1.4) | 1,699 (123) | | 0.320 | 55* | 34* | Mixed competitive | 0.986 |
| | KCl | 8.8 (4.0) | 573 (123) | | 0.120 | 83* | | Noncompetitive | 0.855 |
| | $NH_4Cl$ | 10.9 (7.2) | 515 (176) | | 0.085 | 88 | 29 | Mixed competitive | 0.769 |

[a] The values in parentheses are standard errors.
[b] True first-order rate constants were calculated by linear regression of $CH_4$ oxidation rates against midpoint $CH_4$ concentrations. Pseudo-first-order rate constants were calculated by determining $V_{max}/K_m$ after $K_m$ values were converted to picomoles of $CH_4$ per bottle.
[c] An asterisk indicates that the treatment value was statistically different from the corresponding control value ($P \leq 0.05$).
[d] The regression coefficients are linear for the birch taiga soil and nonlinear (Michaelis-Menten curve fit) for the two temperate soils.

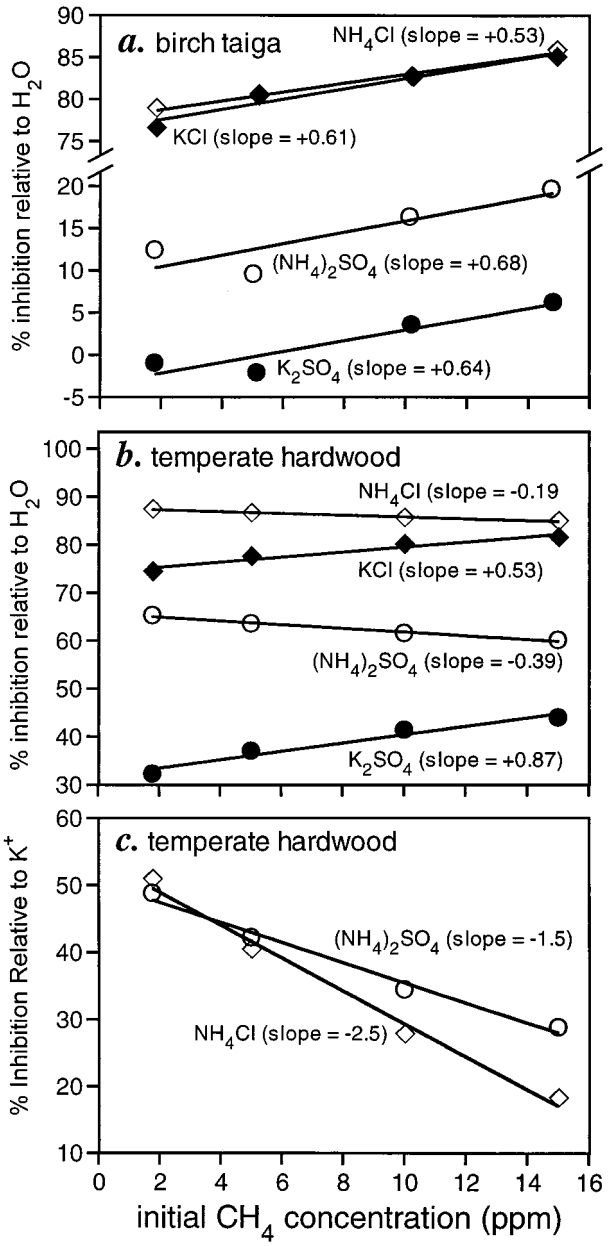


FIG. 2. Effect of $CH_4$ concentration on inhibition of $CH_4$ oxidation. General salt inhibition compared to water controls in the birch taiga soil (a) and in the temperate hardwood soil (b). (c) Specific $NH_4^+$ inhibition compared to $K^+$ controls in the temperate hardwood soil.

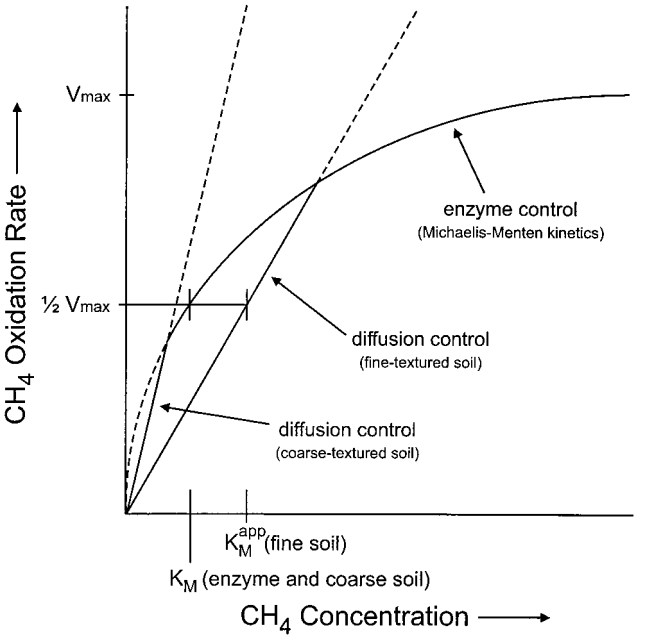


FIG. 3. Potential effect of soil texture on $CH_4$ oxidation kinetics in soil. Because gas transport by diffusion is purely first order, a fine-textured soil may exhibit first-order kinetics at lower $CH_4$ concentrations, whereas a coarse-textured soil containing the same $CH_4$-oxidizing enzyme or a similar enzyme may exhibit Michaelis-Menten kinetics at the same concentrations.

It is unlikely that $K^+$ salts indirectly produced the inhibition observed in this study by desorbing $NH_4^+$ from cation exchange sites, as proposed elsewhere (28). This mechanism requires that an untreated soil contain sufficient exchangeable $NH_4^+$ to account for the inhibition observed with nonammoniacal salts, yet the soils we studied had very low concentrations of exchangeable $NH_4^+$ relative to our $NH_4^+$ additions (Table 1). $Cl^-$ salts consistently inhibit soil $CH_4$ consumption to a far greater extent than do $SO_4^{2-}$ salts (28; this study). King and Schnell (28) attributed this phenomenon to greater $NH_4^+$ adsorption to cation exchange sites in the presence of $SO_4^{2-}$ than in the presence of $Cl^-$. In the present study, however, it was impossible for the KCl treatments to produce free $NH_4^+$ concentrations approaching those of the $(NH_4)_2SO_4$ treatments, because we added 1 to 3 orders of magnitude more $NH_4^+$ than was potentially available in the untreated soils (Table 1). Even so, KCl inhibition was far greater than $(NH_4)_2SO_4$ inhibition in all three soils (Fig. 1; Table 2). KCl and $NH_4Cl$ produced similar levels of inhibition in each of the soils, despite the fact that the $NH_4Cl$ treatments necessarily resulted in much higher free $NH_4^+$ concentrations. Similarly, the results of King and Schnell show that NaCl caused inhibition equal to or greater than the inhibition that equinormal $NH_4Cl$ caused in another temperate forest soil (28). Again, this result could not have been dependent on $NH_4^+$ concentrations. Hence, desorption of endogenous $NH_4^+$ cannot account for the extremely inhibitory effects of $Cl^-$ salts in a variety of soils, and it is clear that $Cl^-$ salts should be avoided in $NH_4^+$ inhibition studies, unless it can be demonstrated that $Cl^-$ is not toxic to $CH_4$ oxidizers in a particular soil.

Unlike KCl, $K_2SO_4$ inhibited $CH_4$ oxidation less than $(NH_4)_2SO_4$ inhibited $CH_4$ oxidation, raising the possibility that there is indirect inhibition by cation exchange when $SO_4^{2-}$ salts are used. Compared to water, $K_2SO_4$ inhibition was 32 to 58% of $(NH_4)_2SO_4$ inhibition in the three soils (Table 2). Assuming that the desorption of endogenous soil $NH_4^+$ was 100%, which is unlikely, the $K_2SO_4$ treatments would have produced maximum $NH_4^+$ concentrations that were between ~0.1 and 10% of the amount added in the $(NH_4)_2SO_4$ treatment (Table 1). Hence, compared to the $(NH_4)_2SO_4$ treatments, the ratios of relative inhibition to potential $NH_4^+$ concentration obtained with the $K_2SO_4$ treatments seem unlikely. More importantly, as discussed below, steady-state kinetic parameters indicate that $K_2SO_4$ and $(NH_4)_2SO_4$ inhibited $CH_4$ oxidation via different physiological mechanisms, which would not be the case if $K^+$ acted indirectly via $NH_4^+$ desorption.

The ubiquity of $NH_4^+$-independent salt effects and the variety of salts that induce similar responses suggest that a fun-

damental physiological process is involved. Roslev et al. (36) found that high-affinity $CH_4$ oxidizers in a temperate forest soil efficiently incorporated $^{14}CH_4$-C into biomass. Adding $NH_4Cl$ to the soil not only decreased $CH_4$ oxidation rates but also reduced the C assimilation efficiency and dramatically increased the proportion of $^{14}C$ oxidized to $CO_2$. It is impossible to know whether this response was to $NH_4^+$ or $Cl^-$ or both, as the experiments did not include parallel salt controls. However, because we found that $Cl^-$ overwhelmingly dominated $NH_4Cl$ inhibition in all of our soils, the response that Roslev et al. observed may also have been predominantly due to $Cl^-$. Killham (25) reported that NaCl additions had the same effect on microbial assimilation and respiration of [$^{14}C$]glucose in soil and found that an increase in the ratio of respired C to assimilated C was a sensitive index of physiological stress within a soil heterotroph community. Shifts in the ratio were attributed to an increase in the maintenance energy required for the cells to cope with the imposed stress. If active transport of ions out of the cell or some other energy-intensive coping strategy were required by energy-limited $CH_4$ oxidizers exposed to a salt, then cellular reductant might be diverted to this process, making less reductant available for growth and potentially to the $CH_4$-oxidizing enzyme, thus decreasing the $CH_4$ oxidation rates. This scenario is plausible for an extremely energy-limited population and is reconcilable with the inhibition kinetics reported here, as diverting reductant away from the $CH_4$-oxidizing enzymes should reduce the catalytic efficiency of the extant enzyme pool, thereby potentially altering $K_{m(app)}$ and $V_{max(app)}$ as described below. Gulledge et al. (17) observed an apparent growth response of the atmospheric $CH_4$ oxidizer community in samples obtained from depths of 20 to 40 cm in another forest soil. The in situ $CH_4$ concentrations at depths below 20 cm were chronically $<0.5$ μl liter$^{-1}$. After 14 days of exposure to ambient atmospheric $CH_4$ in the laboratory, the $CH_4$ consumption rates in water-treated samples increased severalfold compared to the rates measured after only 5 days of exposure. In $K_2SO_4$-treated samples a less pronounced increase occurred, and in $(NH_4)_2SO_4$-treated samples no increase occurred, suggesting that the effects of $NH_4^+$ and salt were synergistic. These results also are consistent with a cellular stress response by an energy-limited population and may illustrate why atmospheric $CH_4$ oxidizers have a limited capacity to recover from soil fertilization (32, 33, 39).

If salts generally inhibit soil $CH_4$ oxidation by an $NH_4^+$-independent mechanism, then it seems appropriate to quantify specific $NH_4^+$ inhibition based on a parallel salt control rather than a deionized water control. This approach has been challenged by the view that other cations may have unique inhibition mechanisms that make them ineffective as experimental controls (28). Although this hypothesis is plausible, no differential toxicity of potential control cations, such as $Na^+$ and $K^+$, has been reported for soil $CH_4$ oxidation. King and Schnell (28) found that KCl inhibited $CH_4$ uptake by pure cultures of *Methylosinus trichosporium* more than did NaCl, but they observed no difference in soil $CH_4$ consumption in the presence of these two salts. Similarly, we observed no difference in the effects of $K_2SO_4$ and $Na_2SO_4$ in the birch taiga soil in the present study (the temperate soils were not tested with $Na^+$). Moreover, it is equally plausible that similar cations, such as $NH_4^+$, $K^+$, and $Na^+$, exert equivalent nonspecific effects that, in conjunction with counteranion effects, account for the nonammoniacal inhibition observed with salts in general. Since $K^+$ and $Na^+$ salts inhibit soil $CH_4$ oxidation to the same extent (28; this study), this hypothesis appears to be sound. Our view, therefore, is that parallel salt controls must be employed when $NH_4^+$ inhibition is examined, because there is no other way to account for the nonammoniacal effects that salts clearly have on soil $CH_4$ consumption. In some cases, salt effects can be substantial compared to specific $NH_4^+$ inhibition and therefore probably interfere with kinetic analysis of the $NH_4^+$ inhibition mechanism. In the present study we used both deionized water and nonammoniacal salt controls in order to examine the relative efficacies of the two approaches for elucidating the mechanism of $NH_4^+$ inhibition.

**Specific $NH_4^+$ inhibition.** Determining the physiological mechanism of specific, immediate $NH_4^+$ inhibition has proven to be difficult (6, 15, 17, 26–28). Dunfield and Knowles (15) demonstrated that $NH_4^+$ inhibited $CH_4$ oxidation by enzymatic substrate competition in an agricultural humisol assayed at high $CH_4$ concentrations. The kinetics varied between samples, however, indicating that an additional mechanism may have been involved. King and Schnell (27, 39) examined $NH_4Cl$ inhibition at low $CH_4$ concentrations and found that relative inhibition increased with $CH_4$ concentration. They concluded that this phenomenon resulted from the fortuitous oxidation of $NH_4^+$ to toxic $NO_2^-$ or $NH_2OH$, which in turn reduced the activity of the methanotroph population (39). They did not examine Michaelis constants or comparable kinetic parameters. We observed similar increases in inhibition with increasing $CH_4$ concentrations in all three of the soils we examined. In the taiga soil, this phenomenon occurred with nonammoniacal salts as well as $NH_4^+$ salts, and the slope of the increase was not affected by the $NH_4^+$ concentration (Fig. 2a). In the temperate soils, $K^+$ salts caused inhibition to increase, whereas $NH_4^+$ salts caused inhibition to decrease as the $CH_4$ concentration increased (Fig. 2b and c). The same pattern occurred whether $Cl^-$ or $SO_4^{2-}$ salts were added, indicating that it was not specific to a particular counterion (Fig. 2a and b). These results indicate that the increase in inhibition did not result from $NH_4^+$ or its by-products. Thus, although $NO_2^-$ undoubtedly inhibits atmospheric $CH_4$ oxidation when it is added directly to soil (19, 26), an increase in $NH_4^+$ salt inhibition when the $CH_4$ concentration increases more likely results from a general salt effect than from by-products of fortuitous $NH_4^+$ oxidation.

A net increase in inhibition with an increase in the $CH_4$ concentration in response to $NH_4^+$ salts may actually indicate that specific $NH_4^+$ inhibition is weak or absent. For instance, in the birch taiga soil, in which $NH_4^+$ inhibition was relatively weak (Table 2), both $NH_4^+$ and $K^+$ salts caused similar increases in inhibition as the $CH_4$ concentration increased (Fig. 2a). However, in the temperate hardwood soil, in which $NH_4^+$ inhibition was relatively strong (Table 2), only $K^+$ salts caused inhibition to increase, whereas $NH_4^+$ salts caused inhibition to decrease as the concentration of $CH_4$ increased (Fig. 2b). Specific $NH_4^+$ inhibition, isolated by using $K^+$ salts as controls, declined precipitously as the $CH_4$ concentration increased (Fig. 2c). Hence, salts generally caused increases in inhibition, whereas $NH_4^+$ caused decreases in inhibition as the $CH_4$ concentration increased, indicating that there are separate inhibition mechanisms for $NH_4^+$ specifically and salts generally. In our soils, the relative strengths of these two mechanisms were apparent from the slopes of the plots of $(NH_4)_2SO_4$ inhibition (relative to deionized water) versus $CH_4$ concentration; a positive slope indicated a stronger salt effect, whereas a negative slope indicated a stronger $NH_4^+$ effect.

The soils which we examined were relatively acidic (pH ~3.5 to 4.5). Because $NH_3$, rather than $NH_4^+$, is probably the competitive inhibitor of $CH_4$ oxidation, salt effects may be more prevalent in acidic soils, whereas competitive inhibition may be relatively more important in neutral to alkaline soils, such as the agricultural humisol investigated by Dunfield and Knowles

(15). Despite the intuitive appeal of this hypothesis, there is no obvious relationship between pH and the degree of $NH_4^+$ inhibition in soils with different pH values, suggesting that other cross-site variables are generally more important (17). Moreover, it is clear from the results obtained with the temperate hardwood soil, in which $NH_4^+$ accounted for 58% of the total inhibition, that $NH_4^+$ inhibition can be dominant in acidic soils. Perhaps the intracellular pH, which should be near neutral regardless of the soil pH, is the relevant control on $NH_3/NH_4^+$ ratios at the enzyme level.

Compared to the $K_m$ in the water controls, the $K_{m(app)}$ either decreased (hardwood soil) or remained unchanged (pine soil) when $K^+$ salts were added, but it always increased when $NH_4^+$ salts were added (Table 2). Again, this pattern supports the hypothesis that there are different inhibition mechanisms for $NH_4^+$ and salts in general and also eliminates the possibility that $K^+$ salts acted indirectly by desorbing soil-bound $NH_4^+$ into solution, as concluded previously for another temperate forest soil (28). If $K^+$ ions acted indirectly via $NH_4^+$, then $K^+$ and $NH_4^+$ salts should have produced similar inhibition kinetics, yet they had different effects on $K_{m(app)}$ compared to deionized water. In contrast to $K_{m(app)}$, $V_{max(app)}$ decreased in response to both $K^+$ and $NH_4^+$ salts (Table 2). Thus, the kinetic constants suggest that there is a partial mixed-type inhibition for $NH_4^+$ salts, with both a competitive component (increasing $K_m$) and a noncompetitive or uncompetitive component (decreasing $V_{max}$) that is independent of $NH_4^+$ (41) (Table 2). If general salt effects account for the additional inhibition, then using $K_2SO_4$ as a control rather than deionized water should isolate the specific $NH_4^+$ effect. Indeed, compared to $K^+$ salts, $NH_4^+$ salts caused $K_{m(app)}$ to increase substantially, whereas they had no effect on $V_{max(app)}$ (Table 2). The $Cl^-$-salt pair produced the same kinetic pattern as the $SO_4^{2-}$ pair despite the greater inhibition by $Cl^-$. These consistent results strongly indicate that $NH_4^+$ inhibited atmospheric $CH_4$ oxidation in the two temperate forest soils via simple enzyme substrate competition.

**Summary and conclusions.** Our approach using $K^+$ salts as controls, and the resulting interpretation, provided a plausible $NH_4^+$ inhibition mechanism that is consistent with the data presented here and can also account for the contrasting results of previous studies (15, 27, 39). Whereas Dunfield and Knowles (15) observed competitive inhibition kinetics in their agricultural humisol, Schnell and King observed increasing inhibition with increasing $CH_4$ concentrations in a temperate forest soil, a result that, by itself, is inconsistent with competitive inhibition. Both phenomena occurred simultaneously in our temperate forest soils and could be explained by a mixed-type inhibition resulting from at least two independent mechanisms, enzymatic substrate competition by $NH_4^+$ and one or more noncompetitive or uncompetitive mechanisms common to salts in general. Although the inhibition mechanism in the birch taiga soil could not be determined directly because it displayed first-order kinetics (Fig. 1a), the relative inhibition pattern for the various treatments was consistent with the patterns obtained with the two temperate soils, so that all three soils may have shared the same mechanisms. Moreover, it is notable that despite very different soil characteristics, we found essentially the same inhibition mechanism that Dunfield and Knowles (15) found in an agricultural humisol. This convergence of physiological responses in ecologically diverse environments suggests that enzymatic substrate competition is an important $NH_4^+$ inhibition mechanism in a wide variety of soils.

Although the results readily explain immediate inhibition of soil $CH_4$ oxidation, delayed inhibition, which has been observed in both field and laboratory studies (17), remains enigmatic. Delayed inhibition probably results from shrinkage of the $CH_4$ oxidizer population over time rather than from decreases in the specific activities of individual $CH_4$ oxidizers (17). $NH_4^+$ and salt effects may act synergistically to impose whole-cell stress that increases maintenance energy requirements, thereby diverting reductant from growth, even if sufficient reductant for the $CH_4$-oxidizing enzyme remains available. This scenario might diminish a population's ability to replace dying biomass, yet might not slow the oxidation of $CH_4$ until the population begins to shrink, resulting in a delayed inhibition response (17). Hence, multiple physiological mechanisms may contribute synergistically to both immediate and delayed $NH_4^+$ fertilizer inhibition of atmospheric $CH_4$ consumption in soil. Moreover, nonammoniacal salts in the environment, especially KCl and NaCl (both of which are used heavily in agriculture and industry), may be as problematic as $NH_4^+$ fertilizers for soil $CH_4$ consumption.

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